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Short Communication

Post-exposure efficacy of Oral T-705 (Favipiravir) against inhalational Ebola virus infection in a mouse model

Sophie J. Smither [a,*], Lin S. Eastaugh [a], Jackie A. Steward [a], Michelle Nelson [a], Robert P. Lenk [b], Mark S. Lever [a]

[a] Biomedical Sciences Department, Defence Science and Technology Laboratory (Dstl), Porton Down, Salisbury, Wiltshire SP4 0JQ, UK
[b] MediVector, Inc., Two International Place, 22nd Floor, Boston, MA 02110, United States

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ABSTRACT

Filoviruses cause disease with high case fatality rates and are considered biological threat agents. Licensed post-exposure therapies that can be administered by the oral route are desired for safe and rapid distribution and uptake in the event of exposure or outbreaks. Favipiravir or T-705 has broad antiviral activity and has already undergone phase II and is undergoing phase III clinical trials for influenza. Here we report the first use of T-705 against Ebola virus. T-705 gave 100% protection against aerosol Ebola virus E718 infection; protection was shown in immune-deficient mice after 14 days of twice-daily dosing. T-705 was also shown to inhibit Ebola virus infection in cell culture. T-705 is likely to be licensed for use against influenza in the near future and could also be used with a new indication for filovirus infection.

Members of the *Filoviridae* family include Ebola virus, Sudan virus, Bundibugyo virus and Marburg virus all of which cause sporadic outbreaks of severe haemorrhagic fever with high case fatality rates (Kortepeter et al., 2011). There are currently no licensed vaccines or therapies against the filoviruses and they are infectious at very low doses. These characteristics mean that the filoviruses are considered possible biowarfare/bioterrorism threat agents that we need to defend against (Bray, 2003; Leffel and Reed, 2004).

Although there are a number of promising vaccine candidates showing efficacy against filoviruses there are less effective post-exposure therapies (Friedrich et al., 2012). Post-exposure therapies in the latter stages of development that show a range of efficacies in primate or rodent models have the disadvantage of being administered by injection (Friedrich et al., 2012). Oral compounds would be a preferred treatment for any therapy, particularly one that may require rapid, wide spread distribution and uptake. Additionally any product would ideally be licensed and have demonstrated safety in humans.

T-705 is a pyrazine compound that inhibits virus RNA polymerase (Furuta et al., 2005). There are a number of recent reports proposing a mechanism of action (Baranovich et al., 2013; Jin et al., 2013; Sangawa et al., 2013). T-705 or Favipiravir (6-fluoro-3-hydroxy-2-pyrazinecarboxamide: $C_5H_4FN_3O_2$) has demonstrated broad spectrum antiviral activity against a number of pathogenic viruses (reviewed in Furuta et al. (2013)). First shown to be effective against influenza viruses (Furuta et al., 2002; Sidwell et al., 2007) it has also been tested against arenaviruses and bunyaviruses (Gowen et al., 2007), West Nile virus (Morrey et al., 2008), Western equine encephalitis virus (WEEV)(Julander et al., 2009a) and more recently has shown *in vitro* inhibition of norovirus replication (Rocha-Pereira et al., 2012).

Favipiravir is now under advanced development for influenza and has completed Phase II and is undergoing Phase III clinical trials (Furuta et al., 2013, http://clinicaltrials.gov/show/NCT01728753). If Favipiravir is licensed for use against influenza, other studies in animal models where Phase III trials are not possible could be used to support the use of the drug against additional RNA viruses as a new indication.

The work presented here shows initial findings of T-705 against Ebola virus (EBOV) *in vitro* and *in vivo* when administered orally in a small animal model.

EBOV E718 (obtained from Public Health England) and EBOV Kikwit (obtained from the Public Health Labs, Canada) were used in the studies. T-705 was provided by the Toyama Chemical Company, Ltd. (Tokyo, Japan) and was made up in 0.5% Carboxymethylcellulose (CMC) (Sigma).

* Corresponding author. Address: Biomedical Sciences Department, Building 7a (Room 201), Defence Science and Technology Laboratory (Dstl), Porton Down, Salisbury, Wiltshire SP4 0JQ, UK. Tel.: +44 (0)1980 614758; fax: +44 (0)1980 614307.
E-mail address: sjsmither@dstl.gov.uk (S.J. Smither).

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Initially, 96-well plates containing confluent monolayers of Vero C1008 cells (ECACC: 85020206) were used to determine cytotoxic levels of T-705. Double dilutions of T-705 starting at 500 mg/ml were performed in duplicate wells. Cytotoxicity in the form of destroyed monolayers was observed at concentrations of T-705 down to 3.9 mg/mL. Monolayers were intact with concentrations of T-705 at 1.95 mg/mL and lower.

To determine the *in vitro* efficacy of T-705, duplicate 96-well plates were coated with confluent Vero C1008 cells at approximately 10^5 cells/well and all cell media was removed. EBOV E718 or EBOV Kikwit was added in Dulbecco's modified Eagles media (DMEM) at an M.O.I. of 0.1 in 100 μL to rows 1–6 of the 8-row plate. T-705 compound at serial 2-fold dilutions starting at 4 mg/mL in 100 μL was added to rows 3–8. Media only was placed in the top 2 and last 2 rows to bring the final volume in all wells to 200 μL and plates were incubated at 37 °C, 5% CO_2. After 6 days, wells were stained with 3 μL Neutral Red (1.5%, Sigma) for 24 h and fixed with 10% formal-saline solution. The plates were then observed for presence or absence of cytopathic effects as an indication of infection, visualised as plaques in the cell layer. The results were the same for both viruses tested and are summarised in Table 1. With addition of T-705 immediately after EBOV was added to cells, infection could be prevented with >6.25 μg of compound (62.5 μg/mL, approximately 400 μM). An EC_{50} or EC_{90} was not determined with the concentration range used but for EBOV is between 31 and 63 μg/mL. This value is higher than that for a range of influenza viruses (<4 μg/mL), arenaviruses (0.5–9 μg/mL) and bunyaviruses (5–30 μg/mL) (summarised in Furuta et al. (2013)), but likely similar to that obtained for WEEV virus (49 μg/mL, Julander et al., 2009a) and yellow fever virus (51.8 μg/mL, Julander et al., 2009b). Cytotoxicity has been shown at a range of higher values (reviewed in Furuta et al. (2013)).

Animal studies were carried out in accordance with the UK Scientific Procedures Act (Animals) 1986 and the UK Codes of Practice for the Housing and Care of Animals Used in Scientific Procedures, 1989, and the US Department of Defense's Animal Care and Use Review Office (ACURO) protocol. Virus-infected animals were housed in rigid-walled isolators held under negative pressure within a dedicated ACDP CL4 animal laboratory and observed twice daily for clinical signs or mortality. Animals were given a week of acclimatisation in the isolator before entering an experiment.

The *in vivo* efficacy of T-705 against wild-type EBOV E718 was determined in a small animal model. A129 interferon alpha/beta receptor$^{-/-}$ knockout immunodeficient mice were used and have previously been shown to be susceptible to a range of wild-type filoviruses (Bray, 2001; Lever et al., 2012). Three groups of six mice were challenged at the same time with a small particle aerosol containing EBOV E718 (determined to be more lethal in A129 knockout mice than Ebola Kikwit (unpublished observation)), as previously described (Lever et al., 2012). EBOV E718 in DMEM at 10^6 $TCID_{50}$/ml was delivered to 18 animals for 10 min. Impinger counts and calculations as previously described (Lever et al., 2012) determined the aerosol concentration to be 3 $TCID_{50}$/L. This resulted in a lethal challenge and calculated dose of ~1 $TCID_{50}$. At 1 h post-challenge and continuing twice daily for 14 days, one group of mice were dosed orally with 150 mg/kg of T-705 in 50 μL 0.5% CMC (3.75 mg per mouse based on a 25 g mouse). Another group were dosed on the same schedule with 0.5% CMC only, and a further group received no treatment, they were challenged with EBOV only. A control group of 6 mice received no challenge but were administered T-705 compound twice a day for 14 days to monitor the effect of the compound only on the animals.

Following EBOV challenge mice that received CMC only or no treatment died 7–8 days later (Fig. 1a) and showed weight loss (Fig. 1b) and clinical signs of severe ruffling, hunched posture and blindness. All mice that were dosed twice daily with T-705 after EBOV challenge showed 100% survival at 4 weeks post challenge, 2 weeks after dosing ended (Fig. 1a). Curve comparison of the test group versus the control group gave $P = 0.009$ using both the Mantel–Cox Log Rank test and the Gehan–Breslow–Wilcoxon test showing that there was a highly significant difference in survival between mice that received T-705 and mice that did not. Surviving mice showed a transient weight loss (Fig. 1b) and mild ruffling of the fur between days 10 and 13 but subsequently recovered to 100% weight and normal appearance. After 30 days the surviving mice were culled and gross pathology appeared normal; there was no difference in appearance of the livers, lungs or spleens between EBOV-challenged/T-705-treated and T-705-treated-only mice. Mice given just T-705 at 150 mg/kg twice a day showed no clinical signs or weight loss over the course of the experiment.

Complete survival against aerosol EBOV infection was achieved with 300 mg/kg/day dosing in an immune-deficient mouse model previously shown to be an appropriate model for predicting virulence of filovirus infections in humans (Lever et al., 2012). This is the first demonstration of the effectiveness of a near-licensed, orally-administered compound against wild-type EBOV infection. The dose used has also been shown to be effective in an arenavirus model against Pichinde virus as a substitute for Lassa or Junin virus (Mendenhall et al., 2011). Dosing with 400 mg/kg/day gave complete protection in a yellow fever hamster model (Julander et al., 2009b), and improved survival in a WEEV mouse model (Julander et al., 2009a). Doses of 100 or 300 mg/kg/day gave high levels of protection against a range of influenza strains (Furuta et al., 2013).

Efficacy has been shown against a high priority threat agent with a compound that is already in the advanced stages of development against influenza. As T-705 is also effective against EBOV there is the potential for a new indication of T-705 when licensed. Future work will assess the efficacy of T-705 against other filoviruses, (most significantly Marburg virus), determine the dose response and effect of delayed time to treatment on survival and test for efficacy against EBOV in a non-human primate model. An efficacious and licensed product for filovirus treatment will have significant impact on global health and defence against bioterrorism.

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JPM-MCS, a component of the Joint Program Executive Office for Chemical and Biological Defense, aims to provide U.S. military forces and the nation with safe, effective, and innovative medical solutions to counter chemical, biological, radiological, and nuclear

Table 1
Activity of T-705 *in vitro*.

T-705 concentration (μg/mL) (2.d.p)	Effect on Vero cells and EBOV (no. wells with CPE/total no. wells tested)
>4000	Toxic to cells
2000	Toxic to cells
1000	Inhibits EBOV (4/4)
500	Inhibits EBOV (4/4)
250	Inhibits EBOV (4/4)
125	Inhibits EBOV (4/4)
62.50	Inhibits EBOV (4/4)
31.25	No effect (0/4)
15.63	No effect (0/4)
7.81	No effect (0/4)
3.90	No effect (0/4)
1.95	No effect (0/4)

CPE: Cytopathic effects.

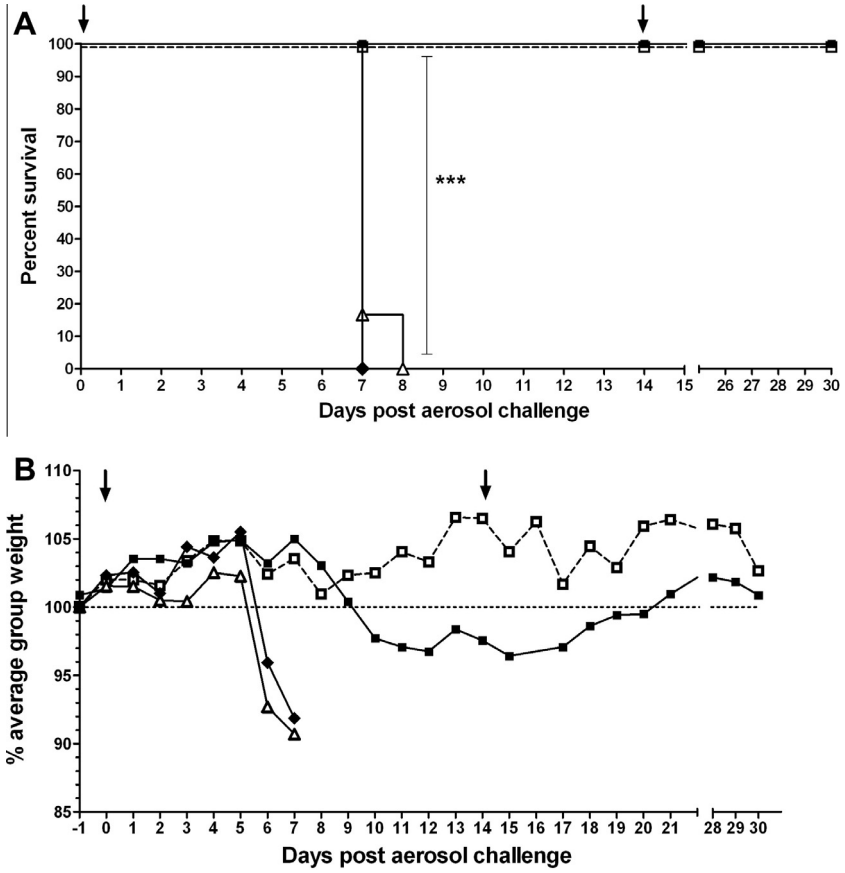

Fig. 1. Survival (A) and weights (B) of A129 Interferon α/β Receptor $^{-/-}$ mice challenged by aerosol with Ebola virus E718 and treated with T-705. Three groups of six mice were challenged with Ebola virus E718 by the aerosol route (solid lines ___). Starting at 1 h post challenge mice received T-705 orally twice a day for 14 days at 150 mg/kg in 0.5% CMC (solid square ■), 0.5% CMC twice a day (open triangle Δ) or no treatment (solid diamond ♦). One group of mice received T-705 treatment but no virus challenge (dotted line - - - , open square □). Arrows indicate the start and end of the dosing period.

threats. JPM-MCS facilitates the advanced development and acquisition of medical countermeasures and systems to enhance our nation' biodefense response capability. For more information, visit www.jpeocbd.osd.mil.

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